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Detect ESBL antibiotic resistance in *K. pneumoniae* and *E. coli* isolates from clinical samples in Al- Basrah governorate, Iraq.

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I. Abstract:

ESBL stands for extended-spectrum beta-lactamases, enzymes produced by some bacteria, making them resistant to many common antibiotics. The most important types of these bacteria are *Escherichia coli* and *Klebsiella pneumoniae*. **Objective**: To detect ESBL antibiotic resistance in isolates of *K. pneumoniae* and *E. coli* from clinical samples in Basrah Governorate. **Methods**: 150 samples were collected from different sources in five hospitals in Basra, located in different cities. 100 samples were collected using swabs and 50 urine samples were transferred to the College of Science, University of Basra, for culture, diagnosis, and detection using drug susceptibility testing. **Results**: Of the 150 samples, 54 (36%) tested positive for K. pneumoniae, and 50 (33.3%) tested positive for *E. coli* on HiCrom selective media. Fifty (92.6%) of the 54 *K. pneumoniae* isolates contained *K. pneumoniae* 16srDNA, while 44 (88%) of the 50 *E. coli* isolates contained the gene. Drug susceptibility testing revealed that 10 (20%) of the *K. pneumoniae* isolates tested positive for extended-spectrum beta-lactamases (ESBLs). 42 (95.5%) of the *E. coli* isolates tested positive for extended-spectrum beta-lactamases (ESBLs). **Conclusion**: A significant increase in the level of resistance of *E. coli* to beta-lactamase antibiotics and the spread of these resistance genes among bacteria poses a threat to global health in Basrah.

Keywords: *antimicrobial-resistant; ESBL; Enterobacteriaceae.*

II. Introduction:

Extended-spectrum beta-lactamases (ESBLs) are enzymes produced by some bacteria (particularly from the Enterobacteriaceae family) that confer resistance to a wide range of beta-lactam antibiotics such as penicillins, cephalosporins (such as ceftriaxone), and aztreonam (Vidal-Cortés *etal*., 2022). Bacteria producing these enzymes are a global threat because the infections they cause are difficult to treat and are associated with increased mortality (Kumar *etal*, 2022). Among members of the Enterobacteriaceae family, *Escherichia coli (E. coli)* and *Klebsiella pneumoniae* (*K. pneumoniae*) are the most clinically significant ESBL producers (Assafi *etal*, 2022).

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Relationship of ESBLs to the Enterobacteriaceae family

The Enterobacteriaceae family includes many pathogenic bacteria capable of acquiring ESBL genes via plasmids or transposons, facilitating their horizontal transmission between bacteria (Assafi *etal*., 2022). ESBLs degrade beta-lactam antibiotics, but they can be inhibited by enzyme inhibitors such as clavulanic acid (which is the basis for their laboratory detection) (Lafta *etal*., 2024). The most important ESBLs in Enterobacteriaceae are: TEM, SHV, and CTX-M: TEM and SHV first appeared in *K. pneumoniae*. CTX-M (particularly CTX-M-15) is the most prevalent worldwide, especially in *E. coli*. Other types (such as OXA, PER, and VEB) are less common but emerging (Verma *etal*., 2023).

E. coli is known for causing urinary tract infections (UTIs), bacteremia, and abdominal infections. Strains producing ESBLs (mostly CTX-M-15) are widespread in the community (such as urinary tract infections in healthy individuals), unlike other hospital-associated ESBLs (Hannoodee & Nasuruddin, 2024). Resistance to antibiotics such as fluoroquinolones (such as ciprofloxacin) and aminoglycosides is common, limiting treatment options (Matinfar *etal*., 2021). Carbapenems (such as meropenem) remain the first choice for severe infections, but resistant strains are emerging. *K. pneumoniae* causes pneumonia, bacteremia, and wound infections, particularly in hospitals and intensive care units (Assafi *etal*, 2022). Most of its ESBL-producing strains used to carry the SHV and TEM genes, but now CTX-M is also prevalent. It is often resistant to multiple antibiotics (such as aminoglycosides and fluoroquinolones) (Raouf *etal*., 2022). Its severity increases when it acquires resistance to carbapenems (such as KPC or NDM strains) (Vidal-Cortés *etal*., 2022). *K. pneumoniae* is characterized as a hospital-associated infection, while *E. coli* is also a community-acquired infection. Excessive use of antibiotics (especially cephalosporins), weakened immunity, and prolonged hospitalization contribute to the development of bacteria carrying ESBL genes (Verma *etal*., 2023). Bacteria carrying ESBL genes can also be diagnosed using a double-disk synergy test or PCR to detect the genes (such as $bla_{CTX\text{-}M}$). Devices such as VITEK and MALDI-TOF accelerate diagnosis (Haudiquet *etal*., 2020).

I. Material and Methods:

1- **Sample collection:** A total of 150 clinical samples were collected between Oct.-2023, 10 Mar.-2024. Samples in this study were included 100 swab samples collected from general wounds, dialysis, abortion, ENT operating rooms, fractures, surgical operations, diabetic foot infections, while 50 urine samples from Urinary tract infection, and cancer patients. These samples were obtained from multiple healthcare facilities in Al-Basrah, including Basrah Oncology Center, Al-Sader Teaching, Al-Basrah Teaching Hospital, Al-Faiha General Hospital, and Al-Mauany Hospital. the samples were collected using a sterile swab with transport media, and used sterile container for urine samples and promptly transferred to the Biology Department at the College of Science, where stored in a preservation box.

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2- **Isolation and Purification of Bacterial Isolates:** All samples were cultured on MacConkey agar (**Karah *etal*., 2020; Viegas *etal.*, 2020**) and incubated at 37°C for 24h. Following incubation, distinct colonies from the MacConkey agar were subculture onto selective media-HiCrome Agar for *K. pneumoniae* and HiCrome Agar for *E. coli*, and incubated at 37°C for 24h. to obtain pure isolates. The purified bacterial colonies were stored in BHI broth supplemented with 15% glycerol for long-term storage.

3- **Molecular Methods:** DNA and plasmid Extraction**;** the Wizard® Genomic DNA purification kit and Pure Yield™ Plasmid Miniprep System (Promega, USA) was used to extract the genomic DNA and plasmid from *K. pneumoniae* and *E. coli*.

4- **16S ribosomal DNA (16SrDNA) and and *malB* region:** the samples isolate (n=100) were reconfirmed identified by using 16SrDNA specific primers for *K. pneumonia* and *malB* region in *E. coli* Table (1). According to (**Liu *et al.*,2008; Tonu *et al.*,2011**).

Table (1): The Primer Sequence Used in current Study.

Type of bacteria	Primer	Sequence of primer	Length (bp)	Product (bp)	Ref.
K. pneumonia	16SrDNA forward	5`-ATTTGAAGAGGTTGCAAACGAT-3`	22	130 bp	Liu *et al.*,2008
	16SrDNA Revers	5`-TTCACTCTGAATTTTCTTGTGTTC-3`	24		
E. coli	16SrDNA forward	5`- GACCTCGGTTTAGTTCACAGA-3`	21	585bp	Tonu *et al.*, 2011
	16SrDNA Revers	5`- CACACGCTGACGCTGACCA-3`	19		

5- **Antimicrobial Susceptibility**: the detection of susceptible bacteria isolated that produce Extended Spectrum β-lactamase enzyme (ESBL) by used Double disk synergy test (DDST) according to (**CLSI ,2020**).

II. Result:

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1- Bacterial Isolation and Identification:

One hundred fifty samples were collected from four different hospitals, with the highest percentage of samples from Basrah Teaching Hospital (45.3%), followed by Al-Sadr Teaching Hospital (28%), and then Al-Faiha General Hospital and Al-Muni Hospital (13.3% each). The samples were divided by source, with urine samples being the most common (33.3%), followed by dialysis samples (23.3%), operating room samples (13.3%), and other sources such as abortion, neurosurgery, diabetic foot, and general surgery. Of the 150 samples, 104 (69.3%) were isolated containing bacteria using HiCrome *K. pneumoniae* agar and *E. coli* HiCrome which these differential media, where 54 (36%) tested positive for *K. pneumoniae*, and 50 (33.3%) tested positive for *E. coli*.

2- Genotypic identification:16S ribosomal DNA (16SrDNA) in *K. pneumoniae* and *malB* gene in *E. coli* PCR Detection:

The DNA extracted was subjected to PCR for amplifying 16S rDNA. In dividual band of 16S rDNA was characterized in (130bp) in *K. pneumoniae* and *malB* gene (585 bp) in *E. coli* by comparison with standard molecular DNA ladder (2000 bp). All isolates performed molecular diagnoses utilizing PCR technique based on the diagnostic gene *16s rDNA*, the results of the current study used n= 54 *K. pneumoniae* isolates the results were shown the 50 (92.6%) *K. pneumoniae* isolates out of 54 *K. pneumoniae*. And had a molecular weight of around 130 bp in comparison to the DNA ladder figure (1), while 4(7.4%) isolates were giving the negative results. While isolates performed molecular diagnoses utilizing PCR technique based on the diagnostic *malB* region, the results of the current study used n= 50 *E. coli* isolates the results were shown the 44(88%) *E. coli* isolates out of 50 *E. coli isolates, and* had a molecular weight of around 585 bp in comparison to the DNA ladder figure (2), while 6(12%) isolates were giving the negative results.

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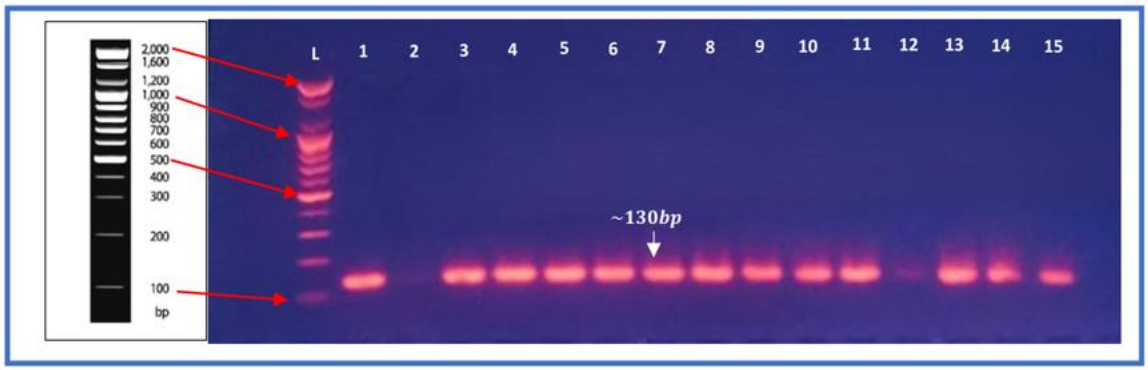

Fig (1) Agarose Electrophoresis Patterns Show PCR Amplified Products of *16s* rDNA. Lane L:(2000 bp DNA ladder), Lanes:(no. 1-15) 16S rDNA band of *K. pneumoniae* isolates. using 1.5% agarose gel, 70V, 45min.

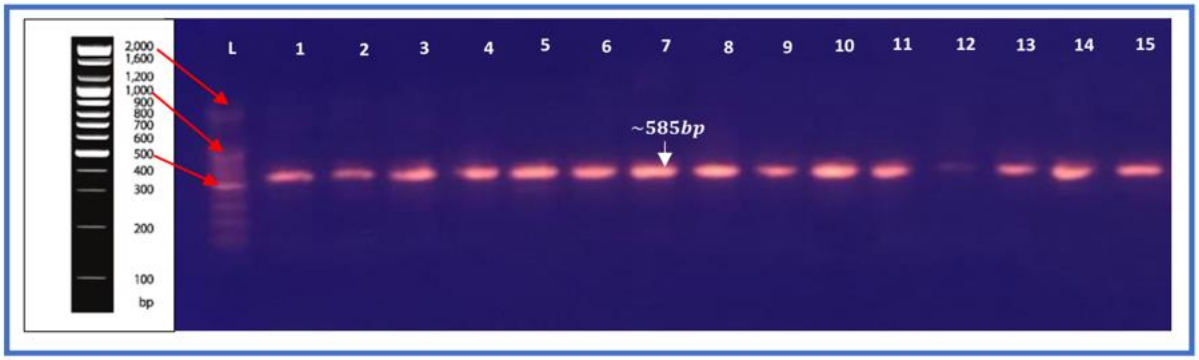

Fig (2): Agarose Electrophoresis Patterns Show PCR Amplified Products of *malB* region. Lane L:(2000 bp DNA ladder), Lanes:(no. 1-15) *malB* region band of *E. coli* isolates. using 1.5% agarose gel, 70V, 45min.

3- Phenotypic Detection of Antibiotic Resistance: Detection of Extended Spectrum β-Lactamase (ESBLs):

In current study that out of n=50 *K. pneumoniae isolates* the only 10(20%) *K. pneumoniae* isolates were gave positive results for produced extended-spectrum β-lactamase (ESBLs). Whereas the 42(95.5%) *E. coli* isolates were giving positive results for produced extended-spectrum β-lactamase (ESBLs). While 40(80%) *K. pneumoniae* isolates and the 2(4.5%) *E. coli* isolates, were

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shown negative results for produced ESBLs by using the double-disc approximation method (DAM), figure (3).

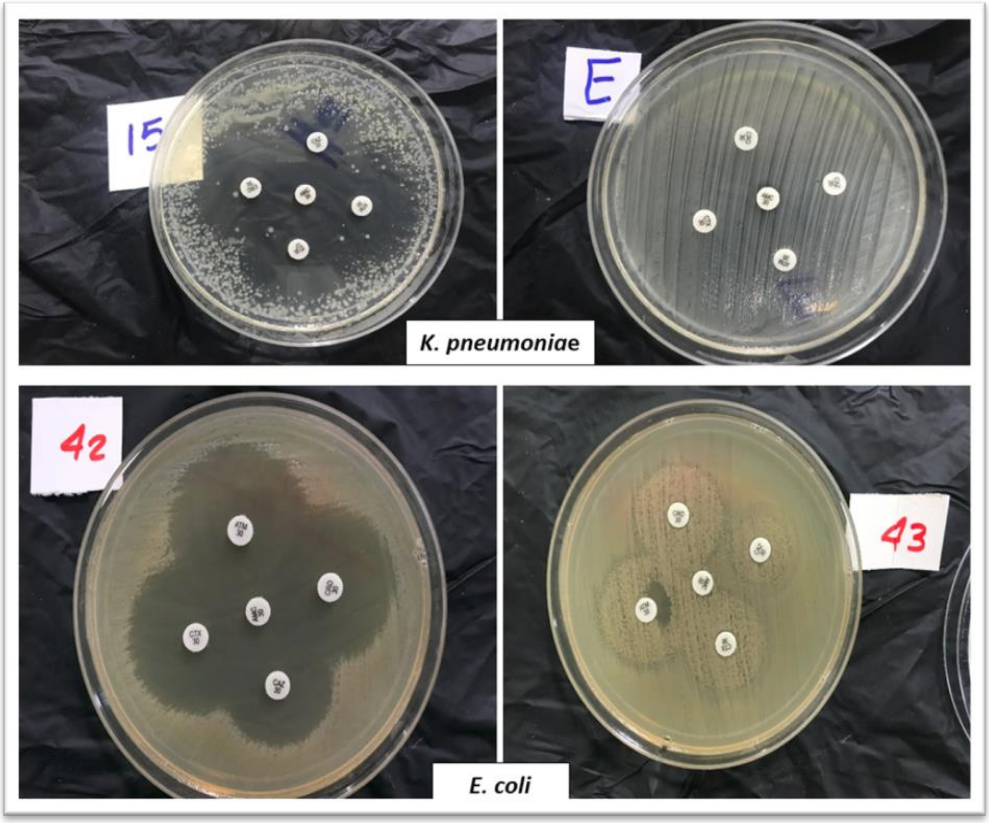

Fig (3): Double-disc approximation (DAM) test that used to detect ESBLs produced by *K. pneumoniae* and *E. coli* isolates.

I. Discussion:

1- Bacterial Isolation and Identification:

Our study is consistent with Assafi *etal*., (2022) & Lafta *etal*., 2024 which proved that urinary tract infections are caused by *E. coli*, and with Mohammed & Anwar, (2022) which explained the spread of *K. pneumoniae* in some Basrah hospitals/ Iraq. And it is inconsistent with Jasim *etal*., (2020), which demonstrated that 80% of infections and inflammation are caused by *K. pneumoniae*. The reasons for this disagree is suggested to be the differences in the regions and

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governorates from which the samples were collected, as well as the differences in sample numbers, and also because the study only studied a specific type of infection and its prevalence.

The distribution of samples indicates that Basrah Teaching Hospital was the primary source of samples (45.3%), which may reflect the hospital's size and the number of cases it receives daily, the density of patients, or the prevalence of infections in these areas. The high percentage of urine samples may also reflect the prevalence of urinary tract infections in the area, which is consistent with previous studies that have identified E. coli as the primary cause of UTIs (Assafi *etal*, 2022). The high percentage of *K. pneumoniae* and *E. coli* in the samples confirms that these two bacteria are major causes of infections in hospitals. These results are consistent with previous studies that have shown that *K. pneumoniae* and E. *coli* are among the most common bacteria in hospital infections, especially in intensive care units and patients with chronic diseases (Bazaid *etal*., 2022). Dialysis and ENT surgeries: The prevalence of *K. pneumoniae*, at 31.4% and 22.4%, respectively, suggests the potential for contamination of the hospital environment or medical instruments, particularly during surgical procedures (Lo *etal*., 2024).

The prevalence of *K. pneumoniae* in abortions, at 18.5%, is due to several factors, including: Genitourinary infections: *K. pneumoniae* can cause urinary tract infections and pyelonephritis in pregnant women. If left untreated, this infection can spread to the upper reproductive tract, leading to intrauterine inflammation, chorioamnionitis, and possibly miscarriage or preterm birth. The inflammatory response resulting from the infection can trigger the release of pro-inflammatory cytokines, which may stimulate uterine contractions and cervical changes, increasing the risk of miscarriage (Vidal-Cortés *etal*., 2022; Hannoodee & Nasuruddin, 2024). Sepsis: In severe cases, *K. pneumoniae* can enter the bloodstream, causing sepsis. This systemic infection can lead to multi-organ failure and disseminated intravascular coagulation (DIC), all of which can threaten the pregnancy and result in fetal loss (Santella etal.,2020). Placental infection: *K. pneumoniae* can infect the placenta, leading to placental insufficiency, fetal abnormalities, and miscarriage. Infection can cause inflammation and necrosis of placental tissue, impeding the transfer of nutrients and oxygen to the fetus (Liu *etal*., 2022; Smith *etal*., 2024).

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Immune suppression: pregnancy is associated with a relatively immunosuppressed state, which may make pregnant women more susceptible to infections, including those caused by *K. pneumoniae*. Infections can be more severe and lead to complications such as miscarriage (Kumar *etal*, 2022).

2- Genotypic identification:16S ribosomal DNA (16SrDNA) in *K. pneumoniae* and *malB* gene in *E. coli* PCR Detection:

The 16s rDNA gene is a suitable target for a variety of molecular investigations because of its properties. The improved identification and detection of bacteria in clinical samples has been greatly supported by 16s rDNA molecular diagnostics. When a bacterial infection is suspected, the most common method used in a typical clinical microbiological laboratory is that method (Harris *et al*., 2003; Church *etal*., 2023). The 16s rDNA gene is found in all bacteria, often in several copies (Kang *etal*., 2010; Schloss, 2021). According to Cheng *etal*. (2018); Church *etal*., (2020) and Chen *etal*., (2023), genotyping is crucial for identifying *K. pneumoniae* cases or outbreaks as well as for identifying the origin and transmission of infections. Since phenotypic approaches are more susceptible to changes in growth conditions, environmental variables, pH, and temperature, genotypic characterization techniques are often more reliable. 16s rDNA is a great diagnostic tool since it offers several advantages over biochemical and phenotypic characterizations, such as the gene's presence in all bacteria and its lack of widespread mutation (Clarridge, 2004; Pei *etal*., 2023). And due to the great importance of 16S rDNA genes, they are a highly conserved region in bacterial genomes, making them a valuable tool for studying the evolutionary relationships between bacteria (Bartoš *etal*., 2024). 16S rDNA enable the classification and identification of bacterial species based on genetic similarities and differences. They also help distinguish closely related species, such as *K. pneumoniae* and *E. coli*, and understand their genetic diversity (Church *etal*., 2020). 16S rDNA gene is also used to study microbial communities in different environments, their ecological roles, and their interactions with other microorganisms (Kumar *etal*., 2021).

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The *malB* region is a region on the *E. coli* chromosome that contains a cluster of adjacent genes called a maltose operon, which plays important roles in the metabolism of sugars (especially starch and complex sugars) (Cho & Misra, 2021; Wu, 2022). The maltose operon region contains several genes, including *malT*, a gene that regulates the expression of other genes in the region. *MalK*, *malG*, and *malF* are genes that encode proteins responsible for the transport and activity of the maltose transporter. The *lamB* gene produces the maltopurin protein, which allows starch molecules to pass through the outer membrane of the bacteria, enabling the bacteria to use maltose and starch as energy and carbon sources. The proteins encoded in this region also transport maltose across cell membranes. The *malB* region is activated when maltose is present in the environment, where the regulator *MalT* binds to the activating substance (maltose) to initiate gene expression (Jeckelmann & Erni, 2020; Abreu *etal*., 2021; Carreón-Rodríguez *etal*., 2023; Pierlé *etal*., 2023). *malB* is considered a genetic marker and diagnostic tool for *E. coli* strains. These genes, *lamB* or *malT*, are used as markers to identify *E. coli* strains specialized in starch metabolism (Osińska *etal*., 2023; Zimoń *etal*., 2024). Some pathogenic *E. coli* strains may carry mutations in these genes, affecting their ability to grow in specific environments (Kinnersley *etal*., 2021; Livnat & Melamed, 2023).

Biochemical tests can be used to detect this region in the laboratory, using maltose-containing media to differentiate *E. coli* strains based on their ability to ferment this sugar. For molecular biology and biotechnology applications, the *malT* promoter (maltose-activated) is used in gene engineering to express proteins upon the addition of maltose (Maffei *etal*., 2021; Iosub *etal*., 2021; Hariharan *etal*., 2023; Hariharan *etal*., 2024; Kim *etal*., 2024). The *lamB* gene has been historically used in gene cloning because it allows the introduction of foreign DNA into bacterial cells. The *malB* system serves as a model for studying nutrient sensing mechanisms in bacteria (Norris *etal*., 2022; Cronan, 2023). Some *malT* or *malK* mutations prevent bacteria from using maltose, impairing their growth in glucose-poor environments by inhibiting the *malB* gene and preventing the production of the maltopurin protein. These mutations have been used in research to understand the mechanisms of antibiotic resistance (Kinnersley *etal*., 2021; Zheng *etal*., 2025).

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3- Phenotypic Detection of Antibiotic Resistance: Detection of Extended Spectrum β-Lactamase (ESBLs):

Resistance to β--lactam antibiotics has spread widely in many countries of the world, but according to different standards, these differences may be due to the quality of personal hygiene and the indiscriminate use of antibiotics, as well as differences in infection control policy and prolonged stay in the intensive care unit, Beta-lactams resistance is a major clinical the problem in treating pneumonia (Raouf *etal*., 2022; Abdul-Wahab *etal*., 2024). The current study showed similarity in the results of various studies conducted in Iraq and other countries, shows that *E. coli* is more resistant than *K. pneumoniae* to ESBL antibiotics. This difference is attributed to several microbiological, genetic and hereditary factors. Raouf *etal*., (2022) his study was conducted in 2020, Al-Najaf City, Iraq, was shown that used microbiological tests on *K. pneumoniae*, the disk diffusion method was used to test antibiotic sensitivity and production of ESBLs was identified using phenotypic and genotypic methods, explanted *K. pneumoniae*, their resistance to many commonly used antibiotics, which is consistent with our current study, also demonstrated the emergence of strains of *K. pneumoniae* carrying ESBL resistance genes, which necessitates the development of a regular surveillance program to prevent the further spread of these isolates in Iraqi healthcare systems. Whereas our study showed high percentage *E. coli* isolates in UTIs patients/ Iraq, this result agrees with Verma *etal*., (2023) used UTIs patients' samples in North India, showed ESBL high resistance in *E. coli* and high percentage in UTIs patients in North India.

The laboratory-based detection of ESBL producers in clinical specimens is highly sensitive and specific by used MHA and antibiotics to detection ESBLs resistance of bacteria, combined with a short reporting time for results, thus decreasing the workload and reducing the need for unnecessary confirmatory tests (Castanheira *etal*., 2021). *E. coli* and *K. pneumoniae* produce ESBLs, the resistance of which is mainly mediated by plasmids. In addition, ESBLs-carrying genes often carry resistance genes such as aminoglycosides, quinolones, aminoglycosides, cotrimoxazole and chloramphenicol, resulting in multi-drug resistance which ESBL-producing

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bacteria associated with multi-drug resistance to other classes of drugs (Al-Mussawi, 2015). Emergence of plasmid-encoded ESBLs is a significant evolution in antimicrobial resistance (Salihu *etal*., 2020), and this result agree with our study.

E. coli is more resistant to ESBLs in many cases due to the diversity of beta-lactamase enzymes it produces, its high capacity to transfer resistance genes via conjugation plasmids, and the transfer of plasmids carrying ESBL genes between strains (Matinfar *etal*., 2021). *K. pneumoniae*, on the other hand, may rely more on vertical gene transfer or integration with other genetic elements. Its wall is also less permeable to antibiotics and is exposed to stronger selective pressures in the intestinal environment, where it lives (an environment rich in antibiotics due to human use) (Haudiquet *etal*., 2020). This makes it more susceptible to evolutionary pressures to develop ESBL resistance. *K. pneumoniae*, on the other hand, is often found in hospitals or outdoor environments contaminated with *K. pneumoniae*, where its resistance to antibiotics is lower due to its limited exposure to antibiotics or the way it acquired genes are passed on from one generation to the next (Adut, 2022).

Conclusion: High percentage resistance antibiotic of *E. coli* and *K. pneumoniae* to ESBL antibiotics, and their widespread prevalence among patients in Basrah Governorate, whether the infection was environmental or through the hospital (hospitalized patients).

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